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Tbx5 and *Tbx4* Are Not Sufficient to Determine Limb-Specific Morphologies but Have Common Roles in Initiating Limb Outgrowth

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Summary

Morphological differences between forelimbs and hindlimbs are thought to be regulated by *Tbx5* expressed in the forelimb and *Tbx4* and *Pitx1* expressed in the hindlimb. Gene deletion and misexpression experiments have suggested that these factors have two distinct functions during limb development: the initiation and/or maintenance of limb outgrowth and the specification of limb-specific morphologies. Using genetic methods in the mouse, we have investigated the roles of *Tbx5*, *Tbx4*, and *Pitx1* in both processes. Our results support a role for *Tbx5* and *Tbx4*, but not for *Pitx1*, in initiation of limb outgrowth. In contrast to conclusions from gene misexpression experiments in the chick, our results demonstrate that *Tbx5* and *Tbx4* do not determine limb-specific morphologies. However, our results support a role for *Pitx1* in the specification of hindlimb-specific morphology. We propose a model in which positional codes, such as *Pitx1* and Hox genes in the lateral plate mesoderm, dictate limb-specific morphologies.

Introduction

Vertebrate forelimbs and hindlimbs are serially homologous structures. Although the limb buds from which they are derived are patterned by common signals during embryonic development, they ultimately form morphologically distinct structures. A question that arises is how cells exposed to common signals can respond differentially and give rise to distinct morphologies.

Vertebrate forelimbs and hindlimbs arise from regions of the lateral plate mesoderm (LPM) at defined locations along the rostral-caudal axis of the embryo. Transplantation experiments in the chick have demonstrated that limb-type specification, the process by which cells of the prospective limb-forming territories are instructed to form either forelimb or hindlimb, occurs prior to the initiation limb bud outgrowth (reviewed in Logan, 2003; Saito et al., 2002; Stephens et al., 1989).

Three genes have been identified that fulfill many of the criteria to be candidates to specify limb-type identity. Two T-box transcription factors, *Tbx5* and *Tbx4*, are expressed in the LPM of either the prospective forelimb or hindlimb region, respectively (Chapman et al., 1996; Gibson-Brown et al., 1996). In addition, a paired-related homeodomain factor, *Pitx1*, is expressed in the prospective hindlimb region but not in the developing forelimb (Lamonerie et al., 1996; Logan et al., 1998; Shang et al., 1997). The limb-type restricted expression pattern of these genes is retained throughout limb development. Misexpression experiments in the chick have suggested that these genes are involved in specification of limb-specific morphologies. Ectopic expression of *Tbx5* in the chick leg bud can induce a partial leg-to-wing transformation. Conversely, misexpression of *Tbx4* in the chick wing bud is able to cause a partial wing-to-leg transformation (Rodriguez-Esteban et al., 1999; Takeuchi et al., 1999). Similarly, misexpression of *Pitx1* in the wing bud leads to the development of limbs with leg-like characteristics (Logan and Tabin, 1999; Szeto et al., 1999; Takeuchi et al., 1999). Accordingly, in *Pitx1* mutant mice, hindlimbs show a loss of hindlimb characteristics (Lanctot et al., 1999; Szeto et al., 1999).

Gene deletion and knockdown experiments have shown that *Tbx5*, *Tbx4*, and *Pitx1* are required for the initiation and/or maintenance of limb bud outgrowth. Functional knockdown of zebrafish *tbx5* results in a failure to initiate pectoral fin bud formation (Ahn et al., 2002). Similarly, all skeletal elements of the forelimb are missing in a limb-restricted *Tbx5* knockout (Rallis et al., 2003). One of the earliest molecular read-outs of limb initiation is the expression of *Fgf10* in the prospective limb fields (Min et al., 1998; Sekine et al., 1999). When *Tbx5* is inactivated, *Fgf10* is never expressed in the prospective forelimb region (Agarwal et al., 2003). *Tbx5* is therefore required for the induction of *Fgf10* in the LPM at prelimb bud stages, which leads to forelimb bud initiation. In $Tbx4^{-/-}$ embryos, induction and initial patterning of the hindlimb appears normal, but it fails to develop further, and *Fgf10* expression is not maintained in the hindlimb bud mesenchyme (Naiche and Papaioannou, 2003). *Pitx1* mutant mouse hindlimbs also display an outgrowth defect, although much less severe than that observed in either *Tbx5* or *Tbx4* mutants. $Pitx1^{-/-}$ hindlimbs are smaller than wild-type, yet the skeletal elements, with the exception of the ilium, are present (Lanctot et al., 1999; Szeto et al., 1999).

We have used loxP/Cre technology in combination with transgenic methods in the mouse to disrupt and replace *Tbx5* function in the forelimb. Our assay involves attempting to rescue the no-forelimb phenotype of the *Tbx5* limb-restricted knockout (Rallis et al., 2003) by expressing either *Tbx4* or *Pitx1*, or both genes simultaneously, in the forelimb-forming region where *Tbx5* function has been specifically deleted. This genetic assay, which we refer to as the limb-rescue assay, allows us to test the properties of these factors in two processes of limb development: (1) initiation of limb outgrowth and (2) specification of limb-specific morphologies. We show that *Tbx4* can replace the function of *Tbx5* and rescue limb outgrowth, whereas *Pitx1* cannot. In contrast to previous chick misexpression studies, *Tbx4*-rescued limbs have a forelimb-like phenotype, suggesting that *Tbx4* alone is not able to dictate hindlimb-specific morphology and that forelimb characteristics can develop in the absence of *Tbx5*. To determine whether *Pitx1* can

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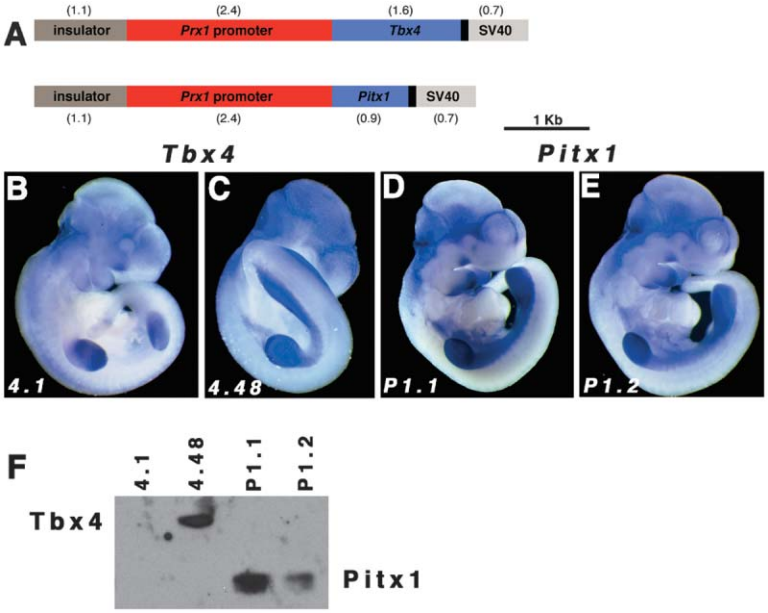

Figure 1. Transgenic Mouse Lines Expressing *Tbx4* or *Pitx1* in the Limbs

(A) Schematic of the *Prx1-Tbx4* and *Prx1-Pitx1* transgenic constructs. The ORF fragments (blue box) contain the HA epitope (black box) as a 3′ fusion. The numbers in brackets denote the size of each fragment in Kbp.

(B–E) Whole-mount in situ hybridization for transgene-derived expression in the developing limbs at E10.5. Expression is also detected in the head and flank in all lines. *Tbx4* expression in *Prx1-Tbx4*(*4.1*) (B) and *Prx1-Tbx4*(*4.48*) (C). *Pitx1* expression in *Prx1-Pitx1*(*P1.1*) (D) and *Prx1-Pitx1*(*P1.2*) (E). Lateral views are shown.

(F) Western blot analysis of protein extracts from transgenic lines. Transgene-derived protein is detected by virtue of the HA tag. In this exposure, protein levels in the *Prx1-Tbx4*(*4.1*) are not detectable. Robust levels of protein are detected in *Prx1-Tbx4*(*4.48*) and *Prx1-Pitx1*(*P1.1*) lines. Lower levels of protein are detected in the *Prx1-Pitx1*(*P1.2*) line.

transform a forelimb to a more hindlimb-like character, we analyzed the morphology of *Pitx1* transgenic forelimbs (expressing endogenous *Tbx5*), and we rescued the no-forelimb phenotype of the *Tbx5* limb-restricted knockout by supplying both *Tbx4* and *Pitx1* simultaneously. In both cases, we observed a partial forelimb-to-hindlimb transformation, suggesting that *Pitx1* does play a role in the specification of hindlimb-specific morphologies but that other factors may also be required.

Results

Generation and Characterization of Transgenic Lines

Conditional deletion of *Tbx5* in the developing limbs leads to the complete absence of all forelimb elements (Rallis et al., 2003). We have exploited this genetic background and developed an assay to test the ability of *Tbx4* and *Pitx1* to rescue the forelimb defect that results from the absence of *Tbx5* function.

To distinguish transgene-derived expression of *Tbx4* and *Pitx1* from endogenous expression, the chick cDNAs of each gene were placed under the regulation of the *Prx1* regulatory element (Martin and Olson, 2000). To enable detection of transgene-derived protein, the cDNAs were tagged with the HA epitope (Figure 1A). Two independent lines were generated for both *Tbx4* and *Pitx1* and denoted *4.1* and *4.48* and *P1.1* and *P1.2*, respectively. In all four lines, the transgene is expressed in the hindlimb and forelimbs as well as the cranial mesenchyme and body wall (Figures 1B–1E), consistent with previous observations of *Prx1*-driven transgenes (Logan et al., 2002). Transgene expression in the limbs corresponds to overexpression of either *Tbx4* or *Pitx1* in the hindlimbs and ectopic expression of these genes in the forelimbs. Western blot analyses and detection with an anti-HA antibody showed differences in the levels of protein expression. The *Prx1-Tbx4*(*4.48*) and *Prx1-Pitx1*(*P1.1*) lines express higher levels of protein than the *Prx1-Tbx4*(*4.1*) and *Prx1-Pitx1*(*P1.2*) lines (Figure 1F).

Tbx4, but Not *Pitx1*, Can Rescue Limb Outgrowth in the Absence of *Tbx5*

Tbx5 is required for initiation and outgrowth of the forelimb (Agarwal et al., 2003; Ahn et al., 2002; Rallis et al., 2003). Using a conditional allele of *Tbx5* and a *Prx1-Cre* deleter transgenic line, we have previously shown that in the absence of *Tbx5* function, the forelimb fails to form (Figure 2B; Rallis et al., 2003). To investigate whether *Tbx4* or *Pitx1* are capable of replacing the function of *Tbx5* in the forelimb, we crossed the *Tbx4* and *Pitx1* transgenic lines into the genetic background of the conditional deletion of *Tbx5* in the limb (*Tbx5*$^{lox/lox}$*;Prx1-Cre*). Heterozygote *Tbx5* mice (*Tbx5*$^{lox/+}$*;Prx1-Cre*), which form normal forelimbs (Rallis et al., 2003), serve as controls (Figure 2A). One of the *Tbx4* lines (*4.48*) was capable of rescuing the forelimb defect in the *Tbx5*$^{lox/lox}$*;Prx1-Cre* mice (Figure 2C), and a limb formed in the forelimb region. This demonstrates that *Tbx4* can replace the function of *Tbx5* in limb outgrowth. The *Tbx4*(*4.1*) line, which expresses much lower levels of Tbx4 protein (Figure 1F), was not able to rescue forelimb development (Figure 2D), suggesting that insufficient amounts of Tbx4 protein are produced. In contrast, none of the *Pitx1*-expressing lines were able to rescue limb outgrowth in the absence of *Tbx5* (Figures 2E and 2F), although at least one line expresses *Pitx1* at higher levels than *Tbx4* in the *Tbx4*(*4.48*) line (Figure 1F). These data demonstrate that *Tbx4*, but not *Pitx1*, can replace the function of *Tbx5* in controlling limb outgrowth.

The *Tbx4*-Rescued Limb Buds Are Normally Patterned

During limb development, a positive feedback loop between *Fgf8*, expressed in the apical ectodermal ridge (AER), and *Fgf10*, expressed in the mesenchyme, is essential for proximodistal outgrowth of the limb bud (reviewed in Martin, 1998). *Shh* expression in cells of the zone of polarizing activity (ZPA) in the posterior limb mesenchyme is required for the precise anterior-posterior patterning of the limb (Riddle et al., 1993). Expression of *Fgf8*, *Shh*, and *Fgf10* in *Prx1-Tbx4*(*4.48*)-rescued

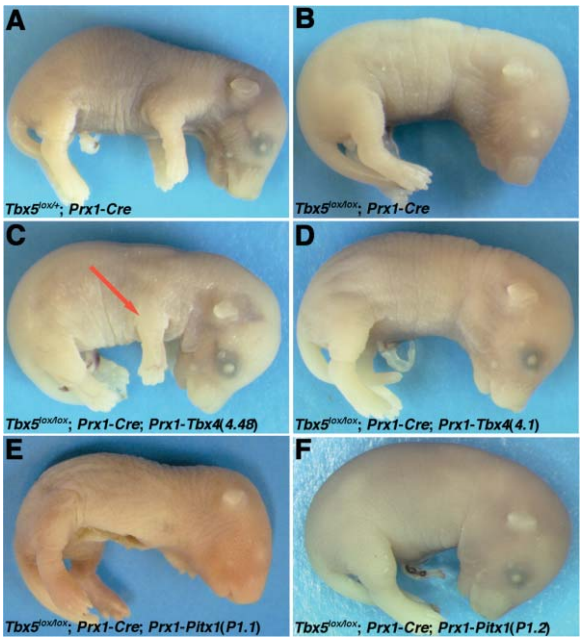

Figure 2. Transgene-Derived *Tbx4*, but Not *Pitx1*, Can Rescue the Limb Defect in the Conditional Deletion of *Tbx5*

All panels show lateral views of E17.5 mouse embryos with the exception of (E), which is P0.

(A) No limb defect is observed following deletion of one copy of *Tbx5* in the limbs.

(B) No forelimbs form following conditional deletion of *Tbx5* in the limbs.

(C) In the absence of *Tbx5*, limb formation is rescued by transgene-derived *Tbx4* using the *Prx1-Tbx4(4.48)* line (arrow).

(D) Limb formation is not rescued by the *Prx1-Tbx4(4.1)* transgenic line.

(E and F) In the absence of *Tbx5*, limb formation is not rescued by *Pitx1* from either the *Prx1-Pitx1(P1.1)* (E) or the *Prx1-Pitx1(P1.2)* (F) lines.

limb buds is identical to that in control littermates (*Tbx5$^{lox/+}$;Prx1-Cre*) (compare Figures 3A with 3D for *Fgf8*, 3B with 3E for *Shh*, and 3C with 3F for *Fgf10*). This demonstrates that in *Prx1-Tbx4*(*4.48*)-rescued limb buds, key signaling centers essential for normal limb development are established and appear to function normally, consistent with our observation that limb outgrowth is rescued at E17.5 (Figure 2C). The failure of Pitx1 and low doses of Tbx4 protein to sustain limb development in *Tbx5$^{lox/lox}$;Prx1-Cre* mice was confirmed in E10.5 embryos. *Fgf8* expression is absent in the forelimb-forming region following attempted rescue with either the *Prx1-Tbx4*(*4.1*), the *Prx1-Pitx1*(*P1.1*), or the *Prx1-Pitx1*(*P1.2*) lines (Figures 3G–3I). Similarly, although expression of *Fgf10* can be detected in the hindlimbs in *Tbx5$^{lox/lox}$;Prx1-Cre;Prx1-Pitx1*(*P1.1*) embryos (Figure 3J), it is absent from the forelimb region at late E9.5 stage. This demonstrates that *Tbx4*, but not *Pitx1*, can initiate and maintain limb outgrowth in the absence of *Tbx5*.

Forelimb-like Identity of the *Tbx4*-Rescued Limbs

Gene misexpression studies in the chick have previously suggested a role for *Tbx5* and *Tbx4* in specification of forelimb and hindlimb identity, respectively (Rodriguez-Esteban et al., 1999; Takeuchi et al., 1999). To test this model using genetic techniques in the mouse, we analyzed the limb-type identity of the *Prx1-Tbx4*(*4.48*)-rescued limb buds and limbs. In addition to *Tbx5*, *Tbx4*, and *Pitx1*, certain Hox genes belonging to the HoxC cluster have a limb-type restricted expression pattern and have been implicated in limb-type specification (Nelson et al., 1996; Peterson et al., 1994). *Hoxc4* and *Hoxc5* are expressed in the forelimb, while *Hoxc9*, *Hoxc10*, *Hoxc11*, *Hoxc12*, and *Hoxc13* are expressed in the hindlimb. Surprisingly, the *Tbx4*-rescued limb has a forelimb-type pattern of gene expression. Following deletion of *Tbx5* and replacement with *Tbx4*, transcripts from the endogenous *Tbx5* conditional allele that has been disrupted by Cre recombinase activity are still detected using a probe that recognizes sequences still present in the recombined, nonfunctional transcript (Figure 4G). Hence, signals that normally restrict *Tbx5* expression to the forelimb (Figure 4A) are still functioning. *Hoxc4* and *Hoxc5*, which are normally expressed in forelimb buds (Figures 4B and 4C), are also expressed in *Prx1-Tbx4*(*4.48*)-rescued limb buds (Figures 4H and 4I). Conversely, genes normally restricted to the hindlimb are not ectopically expressed in the *Prx1-Tbx4*(*4.48*)-rescued limbs. *Tbx4*, *Pitx1*, and *Hoxc10* are expressed in the hindlimbs but not in the *Prx1-Tbx4*(*4.48*)-rescued limb buds (Figures 4J–4L), as seen in control littermates (Figures 4D–4F). In summary, we detect gene expression patterns characteristic of a forelimb in the rescued limb.

To further analyze the identity of the *Prx1-Tbx4*(*4.48*)-rescued limbs, we examined the skeletal morphology in newborn pups and compared them to forelimbs and hindlimbs from control littermates. The forelimb-type character of the rescued limb was evident (compare Figures 4N to 4M and 4O). Three main limb-type-defining features are noticeable: the presence of a scapula, the relative length of the stylopod and zeugopod bones, and the joint between stylopod and zeugopodal elements. The scapula of *Tbx4*-rescued newborn pups is indistinguishable from that found in control littermates (Figure 4N, arrow). The bones of the stylopod and zeugopod articulate to form an elbow-like joint with the distal end of the humerus-like bone sitting in an apparent trochlear notch at the proximal end of the ulna-like bone (Figure 4N, arrowhead). In addition, the relative size of the stylopod and zeugopodal bones is similar. This is most comparable to the arrangement in the forelimb, where the humerus is of equivalent length to the radius and ulna bones while in the hindlimb the femur is smaller than the tibia. Furthermore, both zeugopodal bones in the rescued limb are of similar length, resembling the radius and ulna in forelimbs and not the tibia and fibula in hindlimbs. Although overall the skeletal morphology of the *Tbx4*-rescued limb is remarkably similar to that of the forelimb, it is not completely identical. The deltoid tuberosity of the humerus is absent, and the flexure of the wrist is altered such that the hand extends directly from the wrist and fails to turn inward. In summary, both analyses of *Tbx4*-rescued limbs with limb-type restricted markers at limb bud stages and examination of the limb skeletal morphology in newborn pups demonstrate their forelimb-like phenotype, refuting the postulated role for *Tbx4* in specifying hindlimb identity.

To determine whether these contradictory mouse/chick results were due to differences in the expression

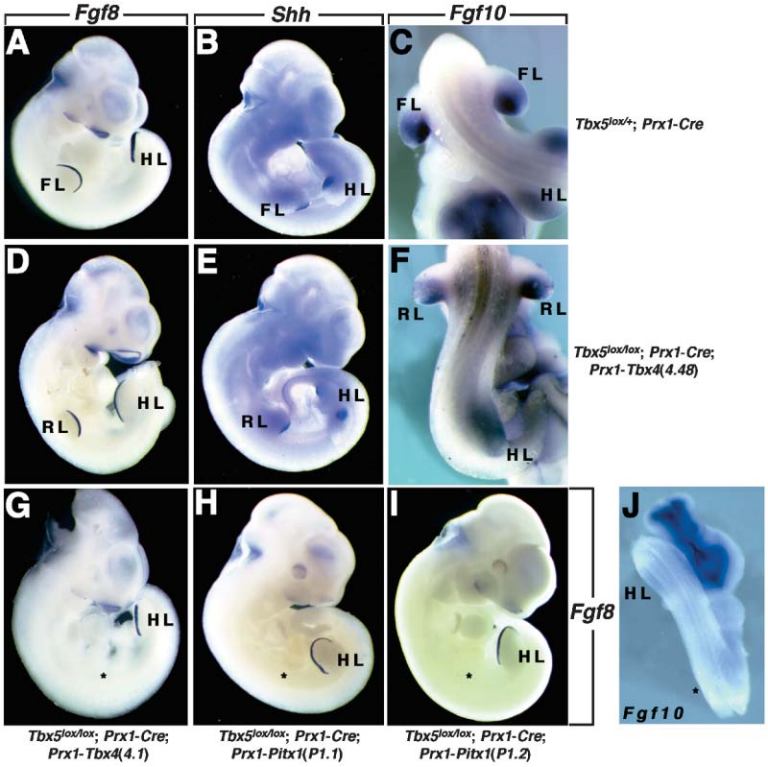

Figure 3. Signaling Centers in the Limb Are Established Normally in *Tbx4*-Rescued Limb Buds

(A–F) *Tbx5*$^{lox/+}$*;Prx1-Cre* control littermates (A–C) and *Tbx5*$^{lox/lox}$*;Prx1-Cre;Prx1-Tbx4(4.48)* embryos (D–F) at E10.5. All are lateral views, except (C) and (F), which are dorsal views. *Fgf8* is expressed in the AER (A), *Shh* in cells of the ZPA (B), and *Fgf10* in the limb mesenchyme (C). *Fgf8* (D), *Shh* (E), and *Fgf10* (F) are all expressed normally in *Tbx5*$^{lox/lox}$*;Prx1-Cre;Prx1-Tbx4(4.48)* embryos.

(G–I) *Fgf8* is not expressed in the forelimb-forming region (indicated with an asterisk) in embryos in which transgene-derived *Tbx4* or *Pitx1* has failed to rescue the limb defect following deletion of *Tbx5*: (G) *Prx1-Tbx4(4.1)*, (H) *Prx1-Pitx1(P1.1)*, (I) *Prx1-Pitx1(P1.2)*.

(J) *Fgf10* is expressed in the hindlimb bud but is not expressed in the forelimb-forming region in which transgene-derived *Pitx1*(*P1.1*) has failed to rescue limb outgrowth (asterisk). FL, forelimb; HL, hindlimb; RL, rescued limb.

levels of *Tbx4*, we doubled the dose of the *Tbx4* transgene by crossing the line to homozygosity in the *Tbx5*$^{lox/lox}$*;Prx1-Cre* background. In these cases, although normal limb morphology is severely affected, forelimb-like characteristics can be still detected. A trochlear notch and an olecranon process in the ulna-like bone are clearly identifiable (Figures 4P and 4Q).

Forelimb-like Identity of *Tbx4*-Rescued Limbs after Ubiquitous Deletion of *Tbx5*

In the *Prx1-Cre* deleter line, Cre activity is first detected at E9.0–E9.5 (Logan et al., 2002). However, *Tbx5* transcripts are first detected at E8.5 (Agarwal et al., 2003). From our previous work (Rallis et al., 2003) and the results presented here for the *4.1*, *P1.1*, and *P1.2* lines that were unable to rescue limb outgrowth (Figure 2), we have demonstrated that this transient expression of *Tbx5* is not sufficient to initiate forelimb development. However, this observation raises the interesting possibility that a short pulse of endogenous *Tbx5* transcript is sufficient to determine forelimb-specific morphology such that, following deletion of *Tbx5* and replacement with *Tbx4*, a forelimb develops. To address this issue, we used the β-*actin-Cre* transgenic line (Lewandoski and Martin, 1997) to disrupt *Tbx5* gene function ubiquitously in the early embryo and tested the ability of the *Prx1-Tbx4*(*4.48*) line to rescue limb outgrowth. Although these embryos die around E10.0 due to heart defects (Bruneau et al., 2001), they survive long enough to determine whether the appropriate set of normally limb-type restricted genes are expressed in these *Prx1-Tbx4*(*4.48*)-rescued limbs.

Analyses of *Tbx5*$^{lox/lox}$*;*β*-actin-Cre;Prx1-Tbx4*(*4.48*) embryos show that *Tbx4* is still able to rescue limb outgrowth even when the cells in the prospective forelimb field have never expressed endogenous *Tbx5* (Figure 5A). Consistent with our results using the *Prx1-Cre* deleter line, these *Tbx4*-rescued limb buds have a forelimb-like gene expression pattern. The normally forelimb-restricted genes *Tbx5*, *Hoxc4*, and *Hoxc5* are expressed in the *Tbx4*-rescued limb in a pattern indistinguishable from wild-type (Figures 5B–5D), while the normally hindlimb-restricted markers *Tbx4*, *Pitx1*, and *Hoxc10* (Figures 5E–5G) are not ectopically expressed. These results demonstrate that *Tbx4* is able to rescue limb outgrowth in cells in the forelimb-forming region that have never been exposed to *Tbx5* activity and that the resultant limb expresses genes normally restricted to the forelimb. Furthermore, genes normally restricted to the hindlimb are not ectopically induced. This demonstrates that an initial pulse of *Tbx5* expression is not able to determine forelimb-specific morphologies.

Pitx1 Can Partially Transform Forelimb to Hindlimb-like Morphologies

Pitx1 is also expressed in a hindlimb-restricted manner and has been implicated in specifying hindlimb-specific morphologies (Lanctot et al., 1999; Logan and Tabin, 1999; Szeto et al., 1999; Takeuchi et al., 1999). *Pitx1*-expressing transgenic lines were not able to rescue limb outgrowth following deletion of *Tbx5*. We were therefore unable to address the ability of this gene to influence limb-type identity in the absence of *Tbx5*. Instead, we have used our transgenic reagents to test the ability of *Pitx1* to transform the forelimb-like morphology of the

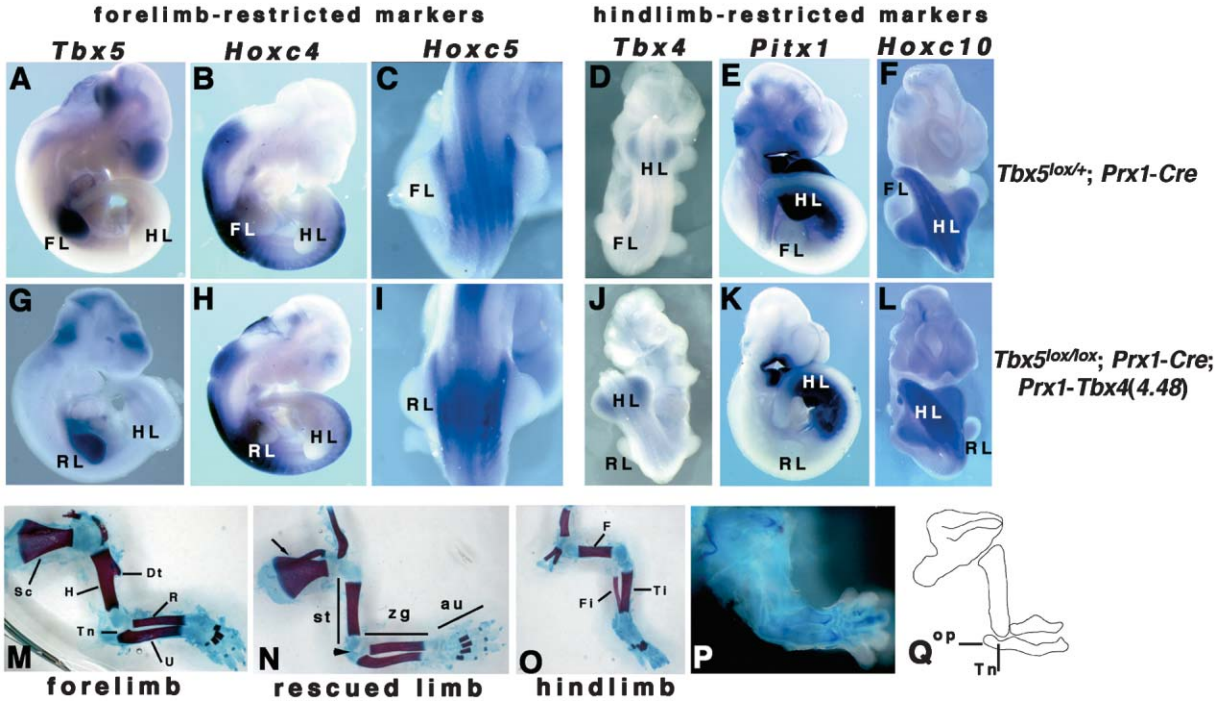

Figure 4. *Tbx4*-Rescued Limbs Have Forelimb Characteristics

Tbx5, *Hoxc4*, and *Hoxc5* are normally expressed in the forelimb but not the hindlimb of control littermates (A–C). They are also expressed in *Tbx4*-rescued limbs (G–I). *Tbx4*, *Pitx1*, and *Hoxc10* are normally expressed in the hindlimb but not the forelimb of E10.5 control embryos (D–F). These genes are not ectopically expressed in the rescued limb at E10.5 (J–L). All the embryos are E10.5 and shown in lateral views except (D), (F), (J), and (L), which are ventral views, and (C) and (I), which are dorsal views. Alcian blue/Alizarin red staining of the control forelimb (M), *Tbx4*-rescued limb (N), and control hindlimb (O) skeletal elements at P0. Alcian blue/Alizarin red staining of a rescued limb (E16.5) in which the *Tbx4* transgene is present to homozygosity (P). An outline of the skeletal elements of the limb shown in (P) illustrates that although abnormal in shape, the articulation is elbow-like (Q). au, autopod; Dt, deltoid tuberosity; F, femur; Fi, fibula; FL, forelimb; H, humerus; HL, hindlimb; op, olecranon process; R, radius; RL, rescued limb; Sc, scapula; st, stylopod; T, tibia; Tn, trochlear notch; U, ulna; zg, zeugopod.

Tbx4-rescued limb and the wild-type forelimb expressing endogenous *Tbx5*.

We generated double-rescued embryos (*Tbx5$^{lox/lox}$;Prx1-Cre;Prx1-Tbx4(4.48);Prx1-Pitx1(P1.1)*) and *Prx1-Pitx1(P1.1)* transgenics and compared their limb skeletal elements to forelimbs and hindlimbs of control littermates (Figure 6) and to the *Tbx4*-rescued limb (Figure 4N). Following deletion of *Tbx5* in cells of the forelimb-forming region and replacement with *Tbx4* and *Pitx1*, the limb element that forms shares many morphological characteristics with a normal hindlimb. The articulation between the stylopod and zeugopod skeletal elements is strikingly knee-like (Figure 6B, arrowhead), while between the zeugopod and autopod it is ankle-like (Figure 6B, double arrowhead) compared to the normal forelimb (Figure 6A). The heads of the stylopod and zeugopod bones in the double rescue limb have a head-to-head apposition and the heads of each bone are larger and broadened, as is found in the knee (Figure 6F, dashed line). Moreover, the double-rescued limb zeugopodal element has a protrusion or tuberosity (Figure 6F, arrow), similar to that observed in the tibia (Figure 6H, arrow) that is not present in the radius (Figure 6E). Similarly, this head-to-head apposition and an extended tuberosity on the zeugopodal bone is present in the *Prx1-Pitx1(P1.1)* transgenic forelimb (Figure 6G, dashed line and arrow, respectively). In addition, at this articulation in the *Prx1-Pitx1(P1.1)* transgenic forelimb, the olecranon process and trochlear notch normally present in the ulna (Figure 6E, arrowhead) are absent (Figure 6G, arrowhead). Digits in the hindlimb are longer than digits in the forelimbs. In the double-rescued and the *Prx1-Pitx1(P1.1)* transgenic limbs, digit lengths are increased and therefore more similar to those in the hindlimb than the forelimb. To quantify these differences, we compared the ratio of the lengths of the second digit metacarpal/metatarsal and first phalange (yellow continuous versus dashed line in Figures 6I–6L). The ratio of metacarpal:phalange length is lower than 2 in the forelimb (Figure 6I, ratio 1.6), while in the hindlimb the second metatarsal is longer than twice the length of the first phalange (Figure 6L, ratio 2.5). In the double-rescued limb, this ratio is also above 2 (Figure 6J, ratio 2.6). Similarly, in the *Prx1-Pitx1(P1.1)* transgenic autopod, the ratio is also above 2 (Figure 6K, ratio 2.25). The length of the anterior zeugopodal bone in the double-rescued and *Prx1-Pitx1(P1.1)* transgenic limbs is longer than the stylopodal bone, resembling the difference in femur/tibia length in the hindlimb rather than the similar length of the humerus/radius of the forelimb.

There are also differences between the *Tbx4*/*Pitx1*-rescued limb and the control hindlimb as well as the *Prx1-Pitx1(P1.1)* transgenic limb. The most obvious is the lack of a posterior zeugopodal bone, with the concomitant loss of the two posterior-most digits. This could be the result of gene dosage effects and may

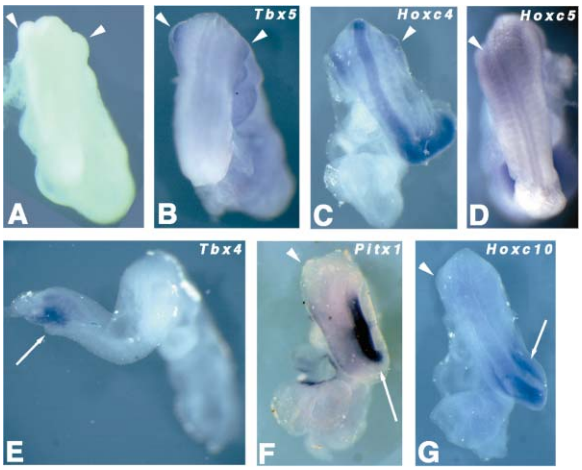

Figure 5. Transgene-Derived *Tbx4* Can Rescue Limb Outgrowth Following Constitutive Deletion of *Tbx5*, and the Rescued Limb Maintains Its Forelimb Identity

All embryos are *Tbx5*$^{lox/lox}$;β-*actin-Cre;Prx1-Tbx4*(*4.48*) at E10 and are shown in dorsal views. *Tbx4*-rescued limbs are indicated with arrowheads.

(A) Rescued limb.

(B–D) Endogenous *Tbx5* (B), *Hoxc4* (C), and *Hoxc5* (D) are expressed in the rescued limb buds.

(E–G) Hindlimb-restricted *Tbx4* (E), *Pitx1* (F), and *Hoxc10* (G) are expressed in the hindlimb (arrows) but not ectopically expressed in the rescued limb.

reveal a role for *Pitx1* and *Tbx4* in regulating cellular responses to signals required for correct anterior-posterior patterning of the limb. In summary, the addition of *Pitx1* into *Tbx4*-rescued limbs or wild-type forelimbs causes a partial forelimb-to-hindlimb transformation, confirming a role for *Pitx1* in specification of hindlimb-specific morphologies. However, these limbs are not entirely transformed to a hindlimb, suggesting that other factors are required to dictate complete hindlimb characteristics and/or that factors in the forelimb region (other than *Tbx5*) prevent complete forelimb-to-hindlimb transformation. Interestingly, *Hoxc10* is not ectopically expressed in *Tbx4*/*Pitx1*-rescued limbs (Figure 6M), indicating that, in this assay, *Tbx4* and *Pitx1* are not sufficient to induce *Hoxc10* expression and that the partial transformation in limb morphology we observe occurs independently of *Hoxc10*.

Discussion

Common Roles for *Tbx5* and *Tbx4* but Not for *Pitx1* during Limb Bud Outgrowth

Our results demonstrate that *Tbx4*, but not *Pitx1*, is able to rescue limb outgrowth in the absence of *Tbx5* (Figure 2), suggesting that *Tbx5* and *Tbx4* play identical biochemical roles in forelimb and hindlimb outgrowth, respectively. In *Tbx4*-rescued limb buds, outgrowth is initiated and the limb bud generated is normally patterned (Figure 3). Consistent with our observations, *Tbx4* can induce the formation of an additional limb when misexpressed in the interlimb flank of a chick embryo (Takeuchi et al., 2003). In contrast, *Pitx1* is not able to induce ectopic limbs when similarly misexpressed (M.P.L., unpublished), consistent with our observation that *Pitx1* alone is not able to rescue limb formation in the *Tbx5* mutant. Furthermore, *Pitx1* null mice hindlimbs, although smaller, possess all the skeletal elements with the exception of the ilium (Lanctot et al., 1999; Szeto et al., 1999), suggesting it has only a minor role in limb outgrowth.

A role for *Tbx5* in initiation of limb outgrowth has been demonstrated in a range of species (reviewed in Logan, 2003). However, a similar role for *Tbx4* in hindlimb initiation has not been demonstrated. In *Tbx4* null mice, hindlimb bud formation is initiated normally, although after E10 further outgrowth is disrupted (Naiche and Papaioannou, 2003). One explanation for the normal initiation of hindlimb development in the *Tbx4* null could be that other factor(s) compensate for the loss of *Tbx4*. We predict that *Pitx1* is not such a compensatory factor because it is not capable of rescuing the *Tbx5* null limb phenotype.

One of the earliest defects in *Tbx5* null mice is the absence of *Fgf10* expression in the LPM, which is required for outgrowth of the limb bud. Moreover, it has been shown that the *Fgf10* promoter contains T-box binding sites and that Tbx5 is able to directly upregulate *Fgf10* expression (Agarwal et al., 2003). The failure to maintain *Fgf10* expression in *Tbx4* mutant hindlimbs suggests that *Tbx4* may also recognize these Tbx binding sites and activate *Fgf10* expression (Naiche and Papaioannou, 2003). Consistent with the idea that *Fgf10* may be a common target of *Tbx5* and *Tbx4*, both genes are necessary to activate the expression of *Fgf10* in the lung mesenchyme (Cebra-Thomas et al., 2003). We predict that in *Tbx4*-rescued limbs, Tbx4 binds to T-box binding sites in the *Fgf10* promoter to activate expression and initiate limb outgrowth.

Tbx5 and *Tbx4* Are Not Sufficient to Determine Limb-Specific Morphology

The limb-type restricted expression patterns of *Tbx5* and *Tbx4* in a range of vertebrate species have suggested that these genes may be involved in an evolutionary conserved mechanism to specify limb-specific morphology (reviewed in Ruvinsky and Gibson-Brown, 2000; Logan, 2003). Moreover, misexpression experiments in the chick led to the conclusion that *Tbx5* and *Tbx4* are sufficient to specify forelimb- and hindlimb-specific morphologies, respectively (Rodriguez-Esteban et al., 1999; Takeuchi et al., 1999).

Our results force a reexamination of the roles of *Tbx5* and *Tbx4* in the specification of limb-specific morphology. In our limb-rescue experiments, although *Tbx4* is able to replace the function of *Tbx5* so that limb outgrowth is maintained, *Tbx4* does not produce a limb with hindlimb-like morphology, and instead the limb elements resemble those of a forelimb. Forelimb-specific genes are expressed in the rescued limb, whereas hindlimb-specific genes are not expressed at any stages analyzed (Figures 4G–4L). Significantly, our results also demonstrate that forelimb morphologies do form in the absence of *Tbx5* and reveal that *Tbx5* is not required for the specification of forelimb-specific morphology. We therefore conclude that *Tbx5* and *Tbx4* do not play

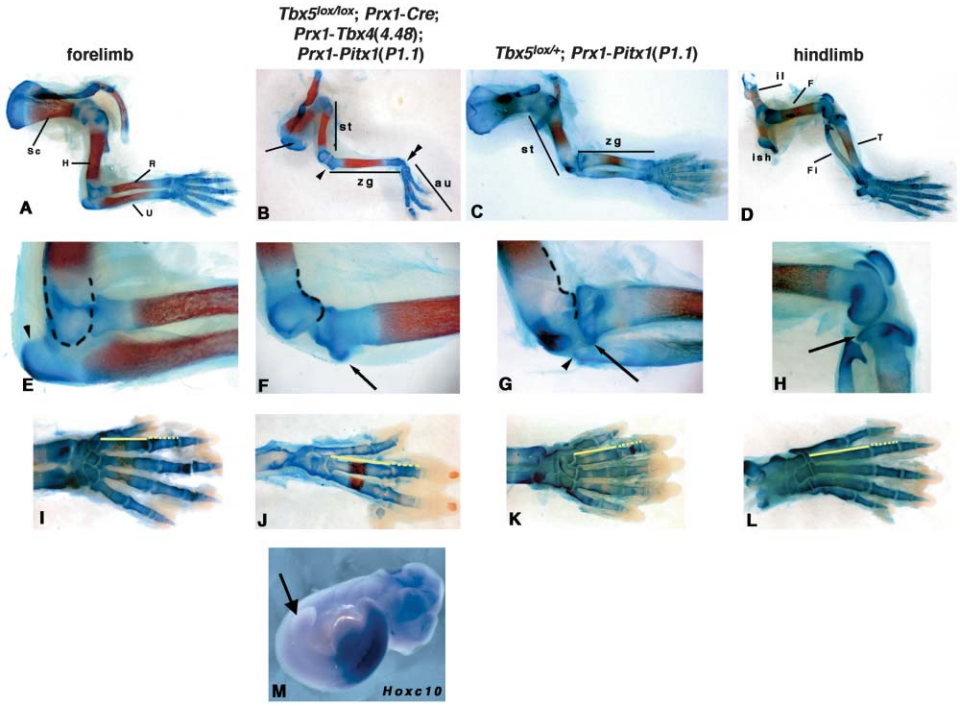

Figure 6. *Pitx1* Transforms the Forelimb-like Morphology of a Normal Forelimb or a *Tbx4*-Rescued Limb to a More Hindlimb-like Morphology

(A–D) Alcian blue/Alizarin red staining of E17.5 control forelimb (A), *Tbx4*/*Pitx1*-rescued limb (B), *Pitx1* transgenic forelimb (C), and control hindlimb (D) skeletal elements.

(E–L) Higher magnifications of the stylopod/zeugopod joint are shown for the control forelimb (E), *Tbx4*/*Pitx1*-rescued limb (F), *Pitx1* transgenic limb (G), and control hindlimb (H) and of the autopod region of the control forelimb (I), *Tbx4*/*Pitx1*-rescued limb (J), *Pitx1* transgenic limb (K), and control hindlimb (L). au, autopod; F, femur; Fi, fibula; H, humerus; ish, ishium; il, ilium; R, radius; Sc, scapula; st, stylopod; T, tibia; U, ulna; zg, zeugopod.

(M) A *Tbx4*/*Pitx1*-rescued embryo at E10.5. *Hoxc10* is expressed in the hindlimb but not ectopically induced in the double-rescued limb bud (arrow).

a role in the specification of limb-specific morphologies but instead have common roles in the initiation and maintenance of limb outgrowth. Reasons for the discrepancies between our results in the mouse and those from misexpression experiments in the chick are unclear. However, we do not believe this is due to differences in expression levels between the two approaches. We have introduced the *Tbx4* transgene to homozygosity in the *Tbx5*$^{lox/lox}$*;Prx1-Cre* background, and these rescued limbs, although morphologically abnormal, are clearly forelimb-like (Figures 4P and 4Q). We suggest that in our transgenic model, we have expressed *Tbx4* at levels appropriate for limb formation and at levels sufficiently above physiological levels such that normal limb formation is disrupted. Even at these higher levels of *Tbx4* expression, we do not detect any apparent transformation of limb-specific morphology.

The conclusions we have drawn from our observations are also consistent with other gene deletion experiments. In *Tbx4* mutant embryos, *Pitx1* continues to be expressed in the hindlimb buds, demonstrating that they retain their hindlimb-like characteristics (Naiche and Papaioannou, 2003). Similarly, a forelimb-to-hindlimb transformation is not observed in *Tbx5*$^{-/-}$ embryos (Agarwal et al., 2003; Rallis et al., 2003). In these embryos, *Tbx5* transcripts are still expressed in the prospective forelimb region, and neither *Tbx4* nor *Pitx1* are ectopically expressed. In addition, transplantation studies in the chick suggest that limb-type identity is specified at stages 9–12, before the induction of *Tbx5* and *Tbx4* in their respective limb fields (Saito et al., 2002; Stephens et al., 1989).

Pitx1 Is a Candidate Axial Cue Required for Specification of Hindlimb-Specific Morphology

Pitx1 is expressed in a broad, caudal domain of the embryo prior to expression of *Tbx4* in the presumptive hindlimb-forming region (Lamonerie et al., 1996; Logan et al., 1998). This appears to be an ancient arrangement that has been conserved during evolution, since the single, ancestral *Pitx* gene is also expressed in a caudal domain in amphioxus (Yasui et al., 2000). In our double-rescued embryos, in which *Tbx5* is replaced with both *Tbx4* and *Pitx1*, and in our *Pitx1* transgenics, the morphology of the resultant limb is significantly more hindlimb-like than the limb element that forms following rescue with *Tbx4* alone. The morphology of the limb elements that form following replacement of *Tbx5* with *Tbx4* and *Pitx1* are not identical to those that form following ectopic expression of *Pitx1* in a forelimb expressing endogenous *Tbx5*. However, we cannot conclude that *Pitx1* is functioning differentially in the presence of either *Tbx4* or *Tbx5*, since the levels of transgene-derived *Tbx4* and endogenous *Tbx5* are, most probably, different. The

differences in levels of transgene-derived *Tbx4* in comparison to endogenous *Tbx5* are apparent since limb elements are absent in *Tbx4*/*Pitx1*-rescued limbs that do form in the *Prx1-Pitx1* transgenic limb. Our results indicate that *Pitx1* is a key downstream factor regulating hindlimb identity. This conclusion is consistent with experiments in the chick that demonstrated that misexpression of *Pitx1* is capable of transforming the wing to a more hindlimb-like structure (Logan and Tabin, 1999; Szeto et al., 1999; Takeuchi et al., 1999) and gene deletion experiments in the mouse in which hindlimb characteristics are lost in *Pitx1* mutants (Lanctot et al., 1999; Szeto et al., 1999).

Our data also suggest that *Pitx1* may not be the single positional cue involved in the regionalization of rostral versus caudal domains in the embryo, subterritories of which will ultimately develop into either a forelimb or a hindlimb, respectively. Replacement of *Tbx5* with both *Tbx4* and *Pitx1* or misexpression of *Pitx1* in a *Tbx5* wild-type background does not produce a complete forelimb-to-hindlimb transformation, presumably due to the absence of other necessary factors or due to the presence of factors that constitute a rostral/forelimb code. Adding support to this model, the limbs that form following replacement of *Tbx5* with *Tbx4* (*Tbx4* rescue) and in *Pitx1*$^{-/-}$ embryos have different morphologies despite both expressing only *Tbx4* and not *Tbx5* or *Pitx1*. In the *Tbx4* rescue, a limb is formed in a rostral (forelimb) territory and has a forelimb-like morphology. In contrast, the limb that forms in a caudal (hindlimb) domain in the *Pitx1*$^{-/-}$ mutant lacks any forelimb characteristics. These results indicate that Pitx1 requires additional factors to distinguish limb-specific morphologies.

Other candidates to specify limb-specific morphologies are the Hox genes. Specific combinations of Hox genes expressed in the embryonic LPM correlate well with the type of limb that will develop (Cohn et al., 1997), and changes in Hox gene expression domains are correlated with the absence of forelimbs in snakes (Cohn and Tickle, 1999). We propose a model (Figure 7) in which limb-specific identity and ultimately limb-specific morphology is specified by different combinatorial codes of factors in the LPM at rostral versus caudal positions. These factors may include a particular combination of Hox proteins and Pitx1. In response to an axial cue that triggers Tbx-mediated limb initiation at the prospective forelimb and hindlimb levels, *Tbx5* is activated as a result of a combinatorial code of "rostral" Hox genes, whereas *Tbx4* expression is initiated by a combinatorial "caudal" Hox code (Ruvinsky and Gibson-Brown, 2000). The activation of both *Tbx5* and *Tbx4* at the prospective forelimb and hindlimb level, respectively, is responsible for the initiation and outgrowth of the limb bud. We conclude that *Tbx5* and *Tbx4* are accurate markers of forelimb and hindlimb identity, respectively, but they do not themselves play a significant role in the specification of limb-type identity.

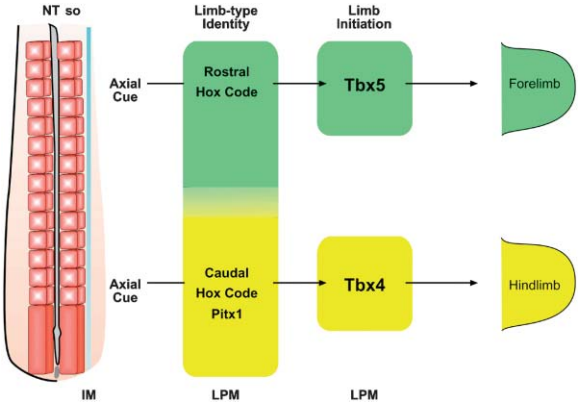

Figure 7. A Model of the Mechanisms that Control Limb-Type Identity and Outgrowth of the Vertebrate Limbs

Broad territories in the flank of the embryo capable of forming either a forelimb or a hindlimb are specified by distinct rostral and caudal combinatorial codes of factors in the LPM. These codes may include a combination of Hox genes in the rostral domain and a combination of Hox genes and *Pitx1* in the caudal domain. In response to a putative axial cue, which may be common to both forelimb and hindlimb, cells in the domain that express a rostral code activate *Tbx5*, which is required for forelimb outgrowth. Cells in the caudal domain, which express a different positional code including *Pitx1*, respond to this axial cue and activate *Tbx4*, which is required for hindlimb outgrowth. IM, intermediate mesoderm; LPM, lateral plate mesoderm; NT, neural tube; so, somite.

Evolution of *Tbx5* and *Tbx4*

Two cognate gene pairs compose the Tbx2/3/4/5 subfamily of T-box transcription factors: the *Tbx2*/*Tbx3* pair and the *Tbx4*/*Tbx5* pair. These gene pairs have evolved from a single ancestral gene by unequal crossing over to form a two-gene cluster that was later duplicated and dispersed in the genome. The evolutionary history of these genes argues for a high degree of functional overlap between cognate genes (Agulnik et al., 1996). Furthermore, the residues that have been shown to be important for DNA binding and dimerization of the T-domain of *Xenopus* Brachyury (the prototype of the T-box family) are identical in mouse and chick *Tbx4* and *Tbx5* (data not shown). Therefore, the simplest model would predict that the Tbx4 and Tbx5 proteins share the vast majority of their target genes rather than having specific targets.

Several models have been proposed to explain the maintenance of duplicate genes in the genome after an initial phase of redundancy. Classical models propose that one of the duplicates will normally degenerate due to the accumulation of deleterious mutations. However, on rare occasions one of the copies may acquire a novel function, endowing the organism with a favorable, selected new function (Ohno, 1970). The duplication-degeneration-complementation (DDC) model was proposed to explain the higher number of duplicate gene copies maintained in duplicated genomes (such as the vertebrate genome) than classical evolutionary models would predict. The DDC model predicts that degenerative mutations in the regulatory elements can increase the probability of duplicate gene preservation and, importantly in our model for *Tbx4* and *Tbx5* function, that the usual mechanism of duplicate gene preservation is the partitioning of ancestral functions rather than the evolution of new functions. We suggest that the function of the *Tbx4*/*Tbx5* gene pair has been partitioned to initiate outgrowth of two sets of serially homologous appendages. Both copies of the gene are then retained

because each duplicate carries out some of the essential functions that were previously accomplished by the ancestral gene (Force et al., 1999). In this example, both *Tbx4* and *Tbx5* would be retained, since each is essential for formation of the hindlimbs and forelimbs, respectively. Regulatory changes, rather than structural changes in the coding region (which tend to be deleterious), have been involved in nonlethal and rapid morphological variation and are therefore candidates to be important components of evolutionary changes. Interestingly, *Pitx1* and *Tbx4* have been involved in macroevolutionary changes related to the morphology of pelvic structures of natural occurring population of three spine sticklebacks (Shapiro et al., 2004).

Experimental Procedures

Generation of Transgenic Lines

The conditional allele of *Tbx5* and *Prx1-Cre* transgenic have been described previously (Bruneau et al., 2001; Logan et al., 2002). *Prx1-Pitx1* transgenic lines were generated by the NICHD Transgenic Mouse Development Facility operated by the University of Alabama at Birmingham. *Prx1-Tbx4* transgenic lines were generated by the Procedural Services section, NIMR. The cDNAs for *Tbx4* (AF033670) and *Pitx1* (AF069397) were HA-tagged at their 3′ end. An insulator element (5′HS4) (Chung et al., 1993) was placed at the 5′ end of the construct. The SV40 polyadenylation signal and artificial intron sequence was placed at the 3′ end of the construct.

Embryos

Mouse embryos were staged according to Kaufman (2001). Noon on the day a vaginal plug was observed was taken to be E0.5 days of development. Mice carrying the conditional *Tbx5* allele, $Tbx5^{lox/lox}$, were identified as previously described (Bruneau et al., 2001). The *Prx1-Cre* transgene was identified as previously described (Logan et al., 2002). The *Prx1-Tbx4* and *Prx1-Pitx1* transgene was identified using a common reverse primer SV40rev and specific forward primers Tb4fwd, Pitxfwd, respectively. The β-*actin-Cre* transgene was identified using *Cre* forward and reverse primers. Details available on request. To generate rescued embryos ($Tbx5^{lox/lox}$*;Prx1-Cre;Prx1-Tbx4*or*Pitx1*), $Tbx5^{lox/+}$*;Prx1-Cre;Prx1-Tbx4*or*Pitx1* mice were crossed to homozygote $Tbx5^{lox/lox}$. To obtain β-actin-Cre-deleted *Tbx4*-rescued embryos, adult heterozygote *Tbx5* mice ($Tbx5^{lox/+}$;β-*actin-Cre*) were crossed to $Tbx5^{lox/lox}$*;Prx1-Tbx4*(*4.48*) mice. Heterozygote *Tbx5* mice ($Tbx5^{lox/+}$*;Prx1-Cre* or $Tbx5^{lox/+}$;β-*actin-Cre*) were used as controls.

Western Blot Analysis

Proteins from both forelimb and hindlimb buds of E10.5 transgenic embryos were obtained using a protein extraction buffer (50 mM Tris-HCl [pH 6.8], 0.5 mM EDTA, 1% SDS). Protein concentration was determined by Bradford method (Bradford, 1976) with a Coomassie protein assay reagent kit (Pierce) with bovine serum albumin as standard protein following manufacturer's instructions. 4 μg of total protein was subjected to standard SDS-PAGE. Proteins were electrotransfered onto the Immun-Blot PVDF membrane (BIO RAD) using a XCell II Blot module (Invitrogen). The membrane was treated with 5% nonfat dry milk in a solution containing 50 mM Tris-HCl (pH 7.4), 0.5 M NaCl, and 0.1% Tween 20 (TBS-T) for 1 hr and then incubated overnight at 4°C with anti-HA rat monoclonal antibody (Roche) at a 1:5000 dilution. Unbound antibodies were washed out with TBS-T. Incubation with peroxidase-conjugated goat anti-rat IgG (Calbiochem) at a 1:5000 dilution was performed at room temperature for 1 hr. Unbound antibodies were washed out with TBS-T. The SuperSignal West Pico Chemiluminescent Substrate (Pierce) was used for HRP detection. The membrane was subsequently exposed to CL-X posure Film (Pierce).

Whole-Mount In Situ Hybridization

Whole mount in situ hybridizations were carried out essentially as previously described (Riddle et al., 1993). All probes have been described previously: c*Tbx4*, c*Pitx1*, m*Tbx4*, m*Tbx5* (Logan et al., 1998), m*Fgf8* (Crossley and Martin, 1995), m*Shh* (Echelard et al., 1993), m*Fgf10* (Bellusci et al., 1997), m*Hoxc4*, m*Hoxc5*, m*Hoxc10* (Burke et al., 1995), and m*Pitx1* (Logan et al., 1998).

Skeletal Preparations

The cartilage and bone elements of E17.5 mouse embryos and newborn pups were stained with alcian blue and alizarin red, respectively, essentially as described (Hogan et al., 1994).

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